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Effect of snuff and nicotine on DNA methylation by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone

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In this study we assayed the effects of snuff and nicotine on the DNA methylation by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a powerful tobacco-specific *N*-nitrosamine. Male F344 rats were pretreated for 2 weeks with either a solution of a snuff extract or 0.002% nicotine in the drinking water. Subsequently, the rats were given a single dose of NNK and the effects of snuff and nicotine on the methylation of guanine by NNK in the DNA of target organs of this carcinogenic nitrosamine were determined. Formation of 7-methylguanine in the liver, nasal mucosae and oral cavity and of O^6-methylguanine in the liver and oral cavity was much lower in the rats pretreated with snuff extract than in those not pretreated. On the other hand, pretreatment of the rats with nicotine had no significant effect on the methylation of DNA by NNK nor on the elimination constants of NNK and its major metabolite 4-(methylnitrosamino)-1-(3-pyridly)-1-butanol.

Introduction

The presence of genotoxic tobacco-specific *N*-nitrosamines (TSNA*) in tobacco and tobacco smoke contributes to the carcinogenic activity of these products. It has been shown that snuff (moist, fine-cut chewing tobacco) is capable of inducing oral cavity tumors in laboratory rats (1,2). Moist snuff manufactured in the United States contains p.p.m. quantities of the TSNA, p.p.b. quantities of benzo[a]pyrene and polonium-210 at the level of pCi (3–5). 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a major TSNA, is a strong carcinogen in rats, mice and hamsters (3–4) and is capable of methylating DNA *in vivo* (6–9).

In one of the bioassays designed to study the induction of oral cavity tumors in male F344 rats by TSNA and snuff, it was observed that the carcinogenic potential of a mixture of *N'*-nitrosonornicotine (NNN) and NNK was suppressed when the agents were administered to the oral cavity together with an extract of snuff (2). The results from this previous study suggest that a component(s) within the snuff extract alters the genotoxic activity of the TSNA. In this study we have determined what changes are observed in the levels of O^6-methylguanine(O^6-MeGua) and 7-methylguanine(7-MeGua) formed by NNK as a result of the pretreatment of animals with a snuff extract. Since nicotine is the major component of the snuff extract, we also determined by effects of nicotine pretreatment on the levels of these methylated guanines as a result of NNK administration.

*Abbreviations: TSNA, tobacco-specific *N*-nitrosamines; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NNN, *N'*-nitrosonornicotine; O^6-MeGua, O^6-methylguanine; 7-MeGua, 7-methylguanine.

In an effort to determine if nicotine pretreatment influences NNK metabolism, the pharmacokinetic properties of NNK during nicotine pretreatment were also determined.

Materials and methods

Preparation of materials for bioassays

An extract was prepared from a leading commerical US moist snuff product, purchased in Westchester County, NY in 1983 and was stored in a cold room (≈ 2°C). The snuff was weighed and mixed with H_2O (1:3.3 w/w). The mixture was mechanically stirred overnight and filtered; the filtrate was lyophilized. The resulting residue, (23% by weight of the moist snuff tobacco) was stored in brown bottles at 0–2°C. The solution of snuff extract was prepared by dissolving 10 g of residue in 20 ml H_2O. A solution of nicotine in drinking water was prepared from 200 mg L-nicotine (distilled under vacuum) in 10 l tap water. This was offered *ad libitum* in brown bottles, which were replaced every fourth day.

Bioassays

Male F344 rats from Charles River Breeding Laboratories (Kingston, NY) were obtained at 6 weeks of age. They were maintained on NIH-07 diet and tap water *ad libitum*. The animals were housed in groups of three in solid bottom polycarbonate cages with hardwood bedding and were kept under standard conditions (22 ± 2°C; 50 ± 10% relative humidity; 12-h light, 12-h dark cycle). At 8 weeks of age, six rats were gavaged twice daily with 0.5 ml of the solution of the snuff extract, Monday through Friday, and once daily on weekends. Six control rats were treated in the same manner with tap water only. After two weeks of extract application, all 12 animals were gavaged once with NNK (0.4 mmol/kg b.w.). The solution of NNK prepared for this purpose contained 13.3 mg/ml and the volumes given per animal ranged from 1.4 to 1.9 ml. The peak of DNA methylation by NNK occurs 4 h after administration (7). Therefore, all animals were killed at that time and the lungs, livers, nasal mucosae and oral cavity tissues were removed. DNA was isolated from the lungs, livers and oral cavity tissues from each rat, and from the pooled nasal mucosae of three rats, as described in the literature (6).

For an evaluation of the effects of nicotine on the DNA methylation by NNK 30 male F344 rats were maintained as previously described, but were given a solution of nicotine (0.002%) in tap water *ad libitum*; 30 control rats were given tap water alone. After two weeks, all animals were injected i.v. with NNK (0.4 mmol/kg b.w.; 4.0% in 0.9% saline). Before injection and at several predetermined intervals thereafter, two or four animals from each group were killed. Blood was removed by cardiac puncture for pharmacokinetic determinations and the lungs, livers, nasal mucosae and oral cavity tissues were excised. The DNA from the lung and liver was isolated from each rat; DNA from the oral cavity tissues and nasal mucosae was pooled from the tissues of two animals.

Blood analysis for NNK and 4(methylnitrosamino-1-(3-pyridy)-1-butanol (NNAL)

The extraction procedures and analyses of blood samples for TSNAs have been previously described (10). In brief, the blood is extracted on a Clin-Elut column and eluted with ethyl acetate. The concentrate of the eluate is analyzed by gas chromatography interfaced with a Thermal Energy Analyzer.

Pharmacokinetic analysis

Concentrations of NNK, and its major *N*-nitroso metabolite, NNAL, in blood were analyzed by the Simulation, Analysis and Modeling (SAAM) computer program (11). Data for NNK best fit a two-term exponential equation and those for NNAL best fit a three-term exponential represented by the equations:

$$C(t) = Ae^{-\alpha t} + Be^{-\beta t}$$

and

$$C(t) = Ae^{-\alpha t} + Be^{-\beta t} + Ne^{-kt}$$

where A, B and N are proportionality constants and α, β and k are rate constants.

DNA isolation

DNA was isolated by the modified Marmur method (12), with slight modifications for DNA from the nasal mucosa, as described previously (6).

Analysis of 7-MeGua, O^6-MeGua and guanine in DNA

The method used to analyze methylated guanines was similar to that described

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previously (13). In brief, isolated DNA was dissolved in sodium cacodylate buffer, pH 7.00, and hydrolyzed at 100°C for 35 min. After cooling to 0°C and centrifugation, the DNA was precipitated by addition of ice-cold 1 N HCl to bring the final concentration to 0.1 N HCl. The precipitated DNA was hydrolyzed at 80°C for 30 min and then cooled to 0°C.

The quantitative analysis was done by h.p.l.c. with u.v. and fluorescence detection. A Partisil 10 SCX column (Whatman, Inc., Clifton, NJ) was eluted with 0.03–0.05 M ammonium phosphate buffer, pH 2.0, at 2 ml/min. Levels of guanine were determined by u.v. detection and levels of 7-MeGua and O^6-MeGua were determined by fluorescence detection with excitation at 286 nm and emission at 366 nm. Comparisons of peak area data to a standard curve generated for each analysis yielded the quantitative data.

Results

Methylation of guanine by NNK has been previously demonstrated (6–9). The examination of the effects of snuff and nicotine on the methylation of guanine by NNK was the purpose of this study. The quantitative data for 7-MeGua and O^6MeGua isolated from liver, lung, nasal mucosa and oral cavity tissues of animals treated with snuff and NNK are summarized in Table I. Levels of O^6-MeGua formed under our experimental conditions in the lung and oral cavity tissues were below the limit of detection for this adduct. The levels of methylguanines in snuff-treated animals are the average from five rats (one animal in this experimental group died upon the last administration of snuff extract). The levels determined in the control animals (gavaged with water only) are the average calculated from six rats. Formation of 7-MeGua in the liver, nasal mucosa and oral cavity tissue was much higher for the control animals than for the snuff-treated animals. 7-MeGua formation in the lung tissue was higher for the snuff-treated animals than for the control animals. The levels of O^6-MeGua detected in the liver and nasal mucosa were also much higher in the animals gavaged with water only. As expected, methylation with NNK occurred most extensively in the nasal mucosa, followed by oral cavity tissue, liver and lung for both groups of animals.

Table II summarizes the extent of DNA methylation by NNK observed 0.5, 1, 4, 12 and 24 h after i.v. injection of the compound. Animals were given tap water *ad libitum* (controls), or nicotine solution (0.002%), 2 weeks prior to the administration of NNK. As in the snuff study, no O^6-MeGua was detected in DNA from lung or oral tissue. However, the DNA methylation in the two groups of animals in this study did not differ significantly. The levels of O^6-MeGua in the lung and nasal mucosa and of 7-MeGua in all of the tissues increased through the 24-h period. The levels of the MeGua adducts in the nasal mucosa begin to drop off by the 24-h point. As in the snuff study, DNA methylation by NNK was most extensive in the nasal mucosa, followed by the liver, oral tissues and lung for the 4-h and 12-h points, but after 24 h, the methylation was most extensive in the liver.

In comparing the pharmacokinetic parameters associated with NNK and NNAL, levels in the circulating blood from the control animals and those pretreated with nicotine were not significantly different in the two groups. The elimination rate constants for the control animals were 1.47 h^{-1} and 1.68 h^{-1}, for NNK and NNAL, while they were 1.60 h^{-1} and 1.70 h^{-1} respectively for the nicotine-pretreated animals.

Discussion

The quantitative comparison of guanine methylation in F344 rats in the snuff study clearly indicates that a component or components within the snuff extract has the ability to alter the extent to which DNA is methylated by NNK in several organs in which NNK is known to induce tumors. The greatest suppression was observed for 7-MeGua formation in the DNA of the nasal mucosa followed by O^6-MeGua in DNA of the nasal mucosa, 7-MeGua

Table I. Levels[b] of 7-MeGua and O^6-MeGua in the liver, lung, nasal mucosa and oral cavity tissues of NNK-treated rats

Treatment	Liver		Lung	Nasal		Oral Tissue
	7-MeGua	O^6-MeGua	7-MeGua	7-MeGua	O^6-MeGua	7-MeGua
Snuff	397 ± 47	36 ± 8	86 ± 20	742[a]	55[a]	374 ± 123
Water	575 ± 34	87 ± 11	62 ± 2	5810[a]	187[a]	1225 ± 286

[a]Data are the average of two determinations from tissues combined from 2 animals.
[b]μmoles per mole guanine ± s.e.

Table II. O^6-MeGua and 7-MeGua levels[b] in excised tissues from nicotine treated rats

Time post-treatment (h)	Liver		Lung	Nasal		Oral tissues
	7-MeGua	O^6-MeGua	7-MeGua	7-MeGua	O^6-MeGua	7-MeGua
Nicotine						
0.5				305[a]	102[a]	
1.0				1650[a]	138[a]	
4.0	911 ± 85	99 ± 3	56 ± 5	2190 ± 125	208 ± 13	462 ± 16
12.0	1120 ± 99	76 ± 9	57 ± 6	2120 ± 240	247 ± 29	430 ± 70
24.0	1710 ± 114	84 ± 9	43 ± 2	1270 ± 45	129 ± 28	626 ± 33
Control						
0.5				190[a]	112[a]	
1.0				1260[a]	107[a]	
4.0	869 ± 62	86 ± 9	62 ± 9	1720 ± 143	197 ± 46	284 ± 25
12.0	1240 ± 146	72 ± 6	49 ± 6	2210 ± 128	223 ± 21	389 ± 120
24.0	2200 ± 168	63 ± 2	35 ± 6	1160 ± 30	175 ± 3	419 ± 8

[a]Data are from the combined tissues of two animals.
[b]μmoles per mole guanine ± s.e.

in oral tissue, O^6-MeGua in hepatic DNA and 7-MeGua in hepatic DNA. A slight increase in 7-MeGua formation in lung DNA was observed for the snuff-treated animals.

These data indicate that components within the snuff extract alter the genotoxic potential of NNK. Nicotine, the major tobacco alkaloid, may act as a competitive inhibitor of the metabolic activation of NNK. Polyphenolic compounds, which are present in snuff at concentrations of 1–5%, have been shown to be inhibitors of tumorigenesis and mutagenesis and may also play a role here (14–15). When rats were pretreated with nicotine, the comparative data on guanine methylation indicated that the alkaloid did not alter the DNA methylation by NNK. Under the experimental conditions used, nicotine does not appear to compete with NNK for metabolic activation. These data suggest that nicotine does not influence α-hydroxylation on the butyl side-chain of NNK which is essential for the generation of an electrophilic intermediate capable of methylating guanine. Our experimental design does not reflect the potential for nicotine to influence the extent of hydroxylation on the *N*-methyl carbon which could yield 4-(3-pyridyl)-4-oxobutyl-diazohydroxide. This reactive intermediate in NNK metabolism is presumed to be reponsible for the formation of alkyl-pyridyl DNA adducts. This is an area which is under study (16).

The results presented suggest that components within the snuff extract alter the levels of methylated guanines in DNA which are formed by NNK. Nicotine is not the component which is responsible for this alteration. Several factors may be responsible for this observed effect. The most obvious is that the formation of these adducts is inhibited due to changes in the metabolic pathways of NNK as a result of snuff extract pretreatment. Secondly, it is also possible that constituents within the snuff extract may affect the necessary enzymes required for the repair of this lesion and may affect the various enzymes differently.

The ratio of O^6-MeGua/7-MeGua is used as an indicator of the rate of O^6-MeGua repair. The ratio in the liver of the animals pretreated with snuff extract and controls is 0.091 and 0.151 respectively. This indicates that repair of O^6-MeGua may be accelerated in the liver of the snuff-pretreated animals. In contrast, the O^6-MeGua/7-MeGua ratio for nicotine-treated animals was very similar to the ratio for the control animals, 0.109 compared to 0.099. This indicates that there is no enhancement of O^6-MeGua repair in the nicotine-treated group. In the nasal mucosa, the ratio of O^6-MeGua/7-MeGua for the snuff-treated and control animals was 0.074 and 0.032 respectively. These data suggest that snuff pretreatment does not accelerate repair of O^6-MeGua to a similar extent in all organs and that factors other than an increase in repair are responsible for the decreased levels of O^6-MeGua in the nasal cavity.

In conclusion, this study has demonstrated that snuff extract contains one or more compounds which alter the methylation of guanine by NNK. Nicotine does not play a role in this alteration of genotoxic activity. Further studies are in progress to examine whether specific components in snuff, such as naturally occurring polyphenols, can alter the *in vivo* methylation of DNA by NNK.

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